A NEW PROCEDURE FOR MOUNTING CLEARED LEAVES USING POLYESTER RESIN

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Summary

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Palaeobotanical studies of leaf floras require detailed comparisons with extant leaves. Techniques for clearing and mounting extant leaves using either sodium or potassium hydroxide or a lacto-phenol solution as a clearing agent have long been known. This paper presents modification of this technique using an hydroxide clearing agent and mounting in polyester resin.

In this procedure leaves are cleared in 15% KOH followed by saturated chloral hydrate. Leaves are then dehydrated, stained with safranin and mounted in Mulford EX-80 polyester resin. Use of this mountant shortens preparation time by several weeks as well as giving superior transparency to specimens. Photographic reproduction using direct printing of specimens placed in an enlarger is also discussed.

Introduction

The vast majority of plant macrofossils collected from Tertiary localities is in the form of compressed or impressed leaves. Identification of these leaves is made difficult by the fact that most extant correlatives are described and identified on the basis of floral parts. To overcome this problem, some palaeobotanists are now studying extant plants in terms of their "leaf architecture" (Hickey 1973). For this type of study, detailed observation of the higher order veins of angiospermous leaves is essential, and many workers (Chandrasekharam 1972[1], Christophel 1973[2], Hickey 1973) have found it advantageous to develop or modify techniques for clearing these leaves.

Of the many techniques developed to cope with this problem (Lersten 1967, Dilcher 1974), most can be divided into two broad categories. The first may be termed the lacto-phenol category, the most recent refinement of which has been presented by Herr (1972). This technique is characterized by rapid clearing (1–5 days) and by preservation of cellular and cuticular detail. Its disadvantages include inapplicability to some leaf forms (e.g. those with extremely tough cuticles or those which are highly tomentose), and extreme difficulty in conversion to a permanent mount.

The second category of techniques, which may be collectively termed the hydroxide category, was used initially for the study of cauline vascular tissue (Foster 1952). It usually combines either sodium or potassium hydroxide treatment with further bathing in chloral hydrate or other bleaching agents. This method is characterized by its relatively long clearing time (four to ten weeks) and frequent loss of cellular and cuticular detail. It has the definite advantages, however, of working on almost all leaf types (given appropriate time) and of being readily combined with many permanent mounting techniques.

The technique presented in this paper is a modification of the hydroxide method. The major drawback of the technique in the past has been the large amount of time needed for the preparations. For example, to prepare a large leaf of *Cinnamomum* takes approximately four weeks in hydroxide, three days in chloral hydrate, one day for staining, and an additional three weeks for mounting and drying. This gives a total time of up to 54 days

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[1] Chandrasekharam, A. (1972).—Megafossil Flora of Genesee, Alberta. Ph.D. thesis, University of Alberta, Canada (unpublished).

[2] Christophel, D. C. (1973).—Fossil floras of the Smoky Tower locality, Alberta. Ph.D. thesis, University of Alberta, Canada (unpublished).

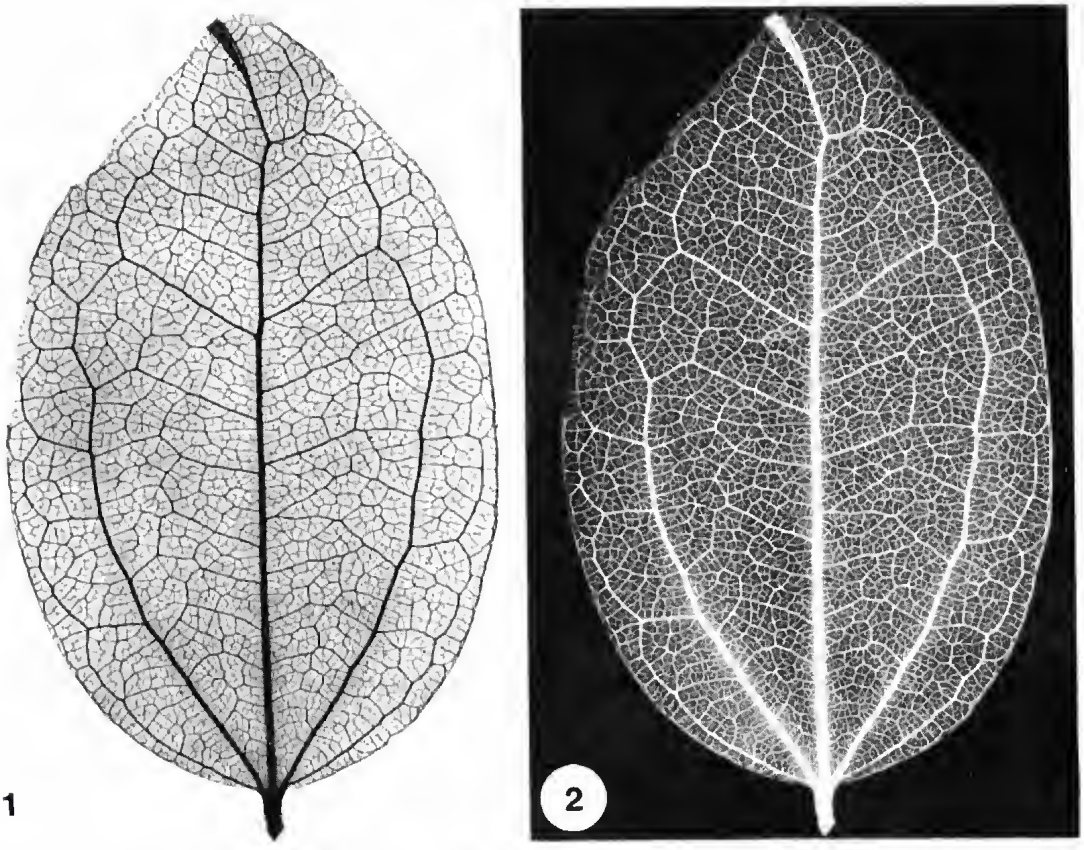

Fig. 1. *Strychnos lucida*—leaf cleared by potassium hydroxide-chloral hydrate method. Photographed with a Leitz Aristophot 4 x 5 plate camera. x 2.5.
Fig. 2. Same specimen as in Fig. 1. Direct print using a Durst 1000 enlarger. x 2.5.

from the date of collection to the date of storage as a permanent mount.

O'Brien (1974) has pointed out that some time may be saved by autoclaving the material in hydroxide. Subsequent to this we have found that the drying time of nearly three weeks for mounting media such as Canada Balsam, Euparal, Permount and Xam can be cut to less than one day by using polyester resin. An added advantage of this procedure is that the monomer of the resin is itself an effective clearing agent. This not only shortens the time needed in clearing, but also produces a specimen with exceptional clarity and reproducibility for photographic work (Figs 1, 2).

Methods

While the basic technique described below was originally outlined by Foster (1952) and incorporates the modifications of others (Chandrasekharam 1972,[1] Hickey 1973), the procedure is presented in its entirety here to facilitate its usage. The method is equally applicable to fresh or dried leaves, with perhaps a slight advantage to the dried material, due to chlorophyll breakdown. All times given are approximate, and will vary with the nature of the material being cleared.

1. Soak leaves in 15% potassium or sodium hydroxide solution until they are decolorised, changing the solution every two days or as necessary. The most satisfactory vessels for this are glass petri dishes, with the leaves placed in them under discs of plastic mesh screening to prevent flotation. The time for decolorisation varies with the specimen from four days to four weeks. Strongly resinous or tanniferous leaves will usually take longest.

2. Wash the leaves under a gentle stream of water and transfer to saturated chloral hydrate until totally clear. This should take from two

to seven days, with slight warming hastening the procedure on slow material.

3. Wash leaves again (as in step two) and transfer to a dehydration series consisting of 10%, 50% and 90% aqueous ethanol. Finally transfer to absolute alcohol. This series should take less than one day.

4. Stain in 1% Safranin in 1:2 absolute alcohol/toluol solution. Stain for three to five minutes or until all vascular tissue has taken up colour.

5. Destain the mesophyll in alcohol/toluol taking care not to destain the ultimate venation. This step determines the final contrast between the mesophyll and the vascular tissue and is consequently critical. Experience has shown that mounted leaves having an absolute absorbance of 1.2 to 2.5 at 525 nm yield the best density for transmitted light photography. Absorbances were measured on a Beckman Acta CIII Spectrophotometer with the leaves at the back of the sample chamber. By comparison, leaves having an absorbance of less than 1.0 proved too light for acceptable reproduction, while those with an absorbance of more than 2.5 proved too dense. As a comparison, the absorbance of 0.2% safranin measured over a 1.0 cm light path is 1.9, and a one cm cuvette of this solution may be used as a control while destaining.

6. Transfer correctly destained leaves to acetone for about ten minutes to remove remaining alcohol/toluol.

7. Without allowing the leaf surface to dry, transfer the leaf to uncatalysed resin monomer[3]. The monomer acts as a final clearing agent and normally renders leaves quite transparent in 30 minutes. Should the leaf surface dry during transfer to the monomer, the resulting opacity may be cleared by leaving the leaf overnight in the monomer. This monomer bath is reusable, and consequently this step is best carried out away from direct sunlight and under fairly cool conditions to prevent polymerisation of the resin.

8. Prepare the final mounting medium by adding about 5% of MEKP catalyst to fresh resin monomer. This is best accomplished by mixing in a small glass phial and rolling between the hands to combine the two components. Such gentle mixing prevents the formation of bubbles in the mountant.

9. Drain the leaf of excess monomer and mount between two glass slides. Setting should take about 2 hours at 30°C. Higher temperatures give shorter setting times but increase the risk of the resin parting from the glass.

10. When the mount is dried, excess resin may be removed with a sharp razor blade or scalpel. Small particles or thin films may be removed with xylene or acetone.

Discussion

Having obtained a permanent mount of a leaf with maximum transparency, it is then necessary to reproduce the venation pattern with as great a degree of contrast as possible for comparison with fossil material. For normal photographs, a Leitz "Aristophot" 4 by 5 sheet film camera with a Macro-Dia attachment for transmitted light gave excellent results (Fig. 1). With a large format camera such as this it is possible to photograph the leaf at nearly life size. The negative can then be contact printed and maximum detail is obtained.

Since maximum contrast between the veins and the mesophyll tissue is desirable, however, the subtle shadings of greys provided by this method proved unnecessary. Galavazi (1965) made brief reference to the direct use of an enlarger for plant material cleared in methyl benzoate. Dilcher (1974) also successfully used this technique on skeletonized and hydroxide cleared leaves. This technique was also admirably suited for our cleared leaves. The mounted leaves fit easily into the negative carrier of a Durst 1000 enlarger and the image can be directly printed on photographic paper. This technique produces a dark-light reversed image (Fig. 2), but as the pattern is the important aspect, this reversal is immaterial.

To achieve maximum contrast, a number of different stains were tried (Morley 1949). Bismarck Brown gave perhaps the greatest contrast, but stained very unevenly. Safranin gave very nearly equal contrast, and had the advantage of staining much more uniformly. Grade four photographic paper was used to give the best contrast in printing.

While the above methods approximately halve the time of the total clearing and mounting procedure, it can still take up to three

[3] For mounting the specimens, Mulford EX-80 polyester resin was used. It is available from Mulford Plastics Pty Ltd., 25 Anzac Highway, Keswick, S. Aust. 5035.

weeks for difficult leaves. Work is in progress using a lacto-phenol clearing method and it is believed that a procedure has now been found to permanently mount specimens cleared in this fashion. Even with this success, however, the hydroxide method, though longer, still appears to give better results with a wider spectrum of leaf types.

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